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<p>(54) Title: AGROBACTERIUM MEDIATED TRANSFORMATION OF GERMINATING PLANT SEEDS</p> <p>(57) Abstract</p> <p>A non-tissue culture process using <i>Agrobacterium</i>-mediated vectors to produce transgenic plants from seeds of such plants as the common bean and soybean.</p>			

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AGROBACTERIUM MEDIATED TRANSFORMATION OF  
GERMINATING PLANT SEEDS  
FIELD OF INVENTION

This invention relates to a process for transforming the  
5 germinating seed of a plant and the use of said process to produce  
transformed plants, particularly dicotyledonous plants.

BACKGROUND OF THE INVENTION

The development of single gene transfer techniques for plant  
species is of great interest and value to plant breeders because it  
10 can be used for the rapid transfer of beneficial genetic traits to  
plants. Numerous methods have been developed for transferring genes  
into plant tissues; Agrobacterium-mediated transfer (Murai et al.,  
1983; Fraley et al., 1983), direct DNA uptake (Paszkowski et al.,  
1984; Potrykus et al., 1985), microinjection (Crossway et al., 1986),  
15 high-velocity microprojectiles (Klein et al., 1987) and electropor-  
ation (Fromm et al., 1985; Fromm et al., 1986). A general problem  
with most of these gene transfer techniques is that the transformed  
tissues, either leaf pieces or cellular protoplast, must be subjected  
to some regeneration steps which require a considerable amount of  
20 time before a whole plant can be obtained. This process is further  
complicated because tissue culture procedures have not been estab-  
lished for many crop species. In most cases, gene transfers into  
crop species have been limited to transformed callus, not whole crop  
plants. In addition, tissue culture procedures can result in  
25 rearrangement of the inserted DNA; or somatic mutations may occur and  
result in the loss or alteration of desirable genetic traits accumu-  
lated by the expertise of many years of plant breeding.

Agrobacterium-mediated gene transfers are by far the most widely  
used gene transfer techniques, but the use of Agrobacterium strains  
30 may be limited because they do not efficiently infect monocotyl-  
edonous cereal crop species. However, recent reports (Hooykaas-Van  
Slogteren et al., 1984; Hernalsteens et al., 1984; Graves and  
Goldman, 1986; Grimsley et al., 1987; Schafer et al., 1987; Bytebier  
et al., 1987) suggest that conditions exist whereby Agrobacterium  
35 strains can bind to monocotyledonous plant cells and transfer their  
T-DNA regions into these cells. Interestingly, the report by Graves  
and Goldman (1986) suggests that Agrobacteria can infect scutellar  
and mesocotyl cells of germinating corn (Zea mays) seeds and that the

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resulting plants are transformed, although these transformed plants will be sectored. This technique suggests that Agrobacterium-mediated gene transfer can be accomplished without the need of any tissue culture intermediate steps. Additional support for the transformation of mesocotyl cells of germinating seeds was obtained by Feldmann and Marks (1987) as they were able to obtain G418 resistant Arabidopsis thaliana plants by co-cultivating germinating seeds with Agrobacteria containing a binary plasmid with a plant expressible neomycin phosphotransferase (NPT) II gene in its T-DNA region.

The development of gene transfer techniques for leguminous plants is of commercial interest because it facilitates the development of new cultivars with improved disease resistance, tolerance to specific herbicides and increased nutritional value. Unfortunately, even though these dicotyledonous species are susceptible to Agrobacterium infections (Facciotti et al., 1985; Owens and Cress, 1985; Byrne et al., 1987), its use for transformation is limited due to the lack of available and efficient regeneration procedures, especially for transformed tissues.

Extension of this technique to germinating seed of leguminous plants such as Phaseolus vulgaris, the common bean, is of great importance because regeneration procedures are not available, let alone the regeneration of transformed undifferentiated tissues.

The development of simple, non-tissue culture dependent methods for transfer, stable integration, and sexual transmission of genetic material into plant species is of great interest and importance. Reports from Graves and Goldman (1986) and Feldmann and Marks (1987) present evidence that transformed whole plants can be obtained via Agrobacterium-mediated transformation of the mesocotyl cells of germinating seeds.

The process of this invention represents (1) an improvement of the Graves and Goldman (1986) technique for the transformation of the seeds of monocotyledonous plants and (2) its extension to dicotyledonous plants.

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#### INFORMATION DISCLOSURE

An G, Watson et al., (1985) New cloning vehicles for transformation of higher plants. EMBO J. 4:277-284.

Byrne M.C. et al., (1987) Strain and cultivar specificity in the

-3-

- Agrobacterium- soybean interaction. Plant Cell Tissue and Organ Culture 8: 3-15.
- Bytebier B. et al., (1987) T-DNA organization in tumor cultures and transgenic plants of the monocotyledon Asparagus officinalis.  
5 Proc. Natl. Acad. Sci. USA 84: 5345-5349.
- Chee P. P. et al., (1986) Expression of a bean storage protein "phaseolin minigene" in foreign plant tissues. Gene 41: 47-57.
- Crossway A. et al., (1986) Integration of foreign DNA following microinjection of tobacco mesophyll protoplasts. Mol Gen Genet  
10 10 202:179-185.
- Facciotti D. et al., 1985) Light-inducible expression of a chimeric gene in soybean tissue transformed with Agrobacterium Biotechnology 3:241-246.
- Feldmann K. A. et al., (1987) Agrobacterium-mediated transformation of germinating seeds of Arabidopsis thaliana: A non-tissue culture approach. Mol Gen Gent 208:1-9.  
15
- Fraley R. T. et al., (1983) Expression of bacterial genes in plant cells. Proc Natl Acad Sci USA 80:4803-4807.
- Fromm M. E. et al., (1986) Stable transformation of maize after  
20 gene transfer by electroporation. Nature 319:791-793.
- Fromm M. et al., (1985) Expression of genes transferred into monocot and dicot plant cells by electroporation. Proc Natl Acad Sci USA 82:5824-5828.
- Graves A. C. F. et al., (1986) The transformation of Zea mays  
25 seedlings with Agrobacterium tumefaciens. Plant Mol Biol 7:43-50.
- Grimsley N. et al., (1987) Agrobacterium-mediated delivery of infectious maize streak virus into maize plants. Nature 325:177-179.
- Hooykaas-Van Slogteren G.M.S. et al., (1984) Expression of Ti plasmid genes in monocotyledonous plants infected with Agrobacterium tumefaciens. Nature 311:763-764.  
30
- Hernalsteens J.P., et al, (1984) An Agrobacterium-transformed cell culture from the monocot Asparagus officinalis. EMBO J 3:3039-3044.
- Jefferson (1986) B-Glucuronolose from Escherichia coli as a gene fusion marker. Proc. Natl. Acad. Sci., USA 83:8447-8451.  
35
- Klein T.M. et al.; (1987). High-velocity microprojectiles for delivering nucleic acids into living cells. Nature 327:70-73.
- Murai N. et al., (1983) Phaseolin Gene from Bean is Expressed

-4-

after transfer to Sunflower via Tumor-inducing Plasmid Vectors. Science 222:476-482.

Owens L.D. et al., (1985) Genotypic variability of soybean response to Agrobacterium strains harboring the Ti or Ri plasmids.

5 Plant Physiol 77:87-94.

Paszkowski J. et al., (1984) Direct gene transfer to plants. EMBO J 3:2717-2722.

Pedersen K. et al., (1986) Sequence analysis and characterization of a maize gene encoding a high-sulfur zein protein of MW 10 15,000. J. Biol Chem 261:6279-6284.

Potrykus I. et al., (1985) Direct gene transfer to cells of a graminaceous monocot. Mol Gen Genet 199:183-188.

Reiss B. et al., (1984). A new sensitive method for qualitative and quantitative assay of neomycin phosphotransferase in crude cell 15 extracts. Gene 30:211-218.

Schafer W. et al., (1987). T-DNA integration and expression in a monocot crop plant after induction of Agrobacterium. Nature 328:539-532.

20 Slightom J.L. et al., (1983). Complete nucleotide sequence of a French bean storage protein gene. Phaseolin. Proc Natl Acad Sci USA 80:1897-1901.

A non-tissue culture approach for preparing transformed Arabidopsis thaliana seeds is described by Feldmann and Marks, Mol. Gen. Genet. (1987) 208:19. However, to the inventors' knowledge the 25 application of non-tissue culture transfer has not been successfully applied to leguminous plants and other large seed dicots such as soybean, the common bean, squash, zucchini, peppers, and others.

#### SUMMARY OF THE INVENTION

The present invention provides:

30 A process for producing a transgenic plant which comprises:

(a) germinating a seed of a plant;

35 (b) inoculating the meristematic or mesocotyl cells produced during germination, prior to their differentiation, with a virulent or non-virulent Agrobacterium strain containing a transferable gene in an Agrobacterium derived vector; and

(c) allowing the cells to differentiate into mature plants, with the proviso that the plant cannot be from the family Arabidopsis thaliana.

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The time of infecting germinating *P. vulgaris* seed after germination with the Agrobacterium-based vectors has been found to be critical. The length of time the seeds are allowed to germinate prior to Agrobacteria infection will greatly affect the ability of the Agrobacteria to infect meristematic cells, because the amount of vascular tissue is rapidly increasing as differentiation proceeds. However, seed germination must take place in order to have physical access to the mesocotyl region. Therefore a preferred manner of practicing the invention is to conduct the inoculation step within 16 to 96, preferably 24 to 48, hours of germination. To determine the optimum time for infecting germinating seeds, inoculations with virulent Agrobacterium strain A208, were done at various times after initiating germination, between 6 to 96 hours. Successful transformation was scored by gall formation on the developing seedlings, the results of inoculating 50 seeds for each time interval is presented in Table I. Seeds allowed to germinate between 24 to 48 hours were found to be the most susceptible to Agrobacterium infections. Between 70% to 80% of these inoculated seeds gave rise to seedlings with galls formed either on the hypocotyl, epicotyl, cotyledonary node, or distributed throughout the base of the plant. A preferred method of inoculation is with a virulent or non-virulent Agrobacterium strain containing a transferable DNA cis or trans plasmid.

A particularly preferred manner of practicing the process on dicots involves removing one of the cotyledons prior to inoculation. This step increases access of the strain to the mesocotyl region wherein the meristematic cells are generated.

The method of this invention is simple, rapid, avoids the use of any tissue culture techniques, and transformed plants can be obtained directly.

Also provided are:

Transgenic plants prepared by the process of this invention. Preferred are dicotyledonous transgenic plants. Especially preferred are dicotyledonous plants of the family leguminosae, such as phaseolus vulgaris and Glycinus max.

#### 35 DESCRIPTION OF THE PREFERRED EMBODIMENT

Germinating seeds are inoculated with either virulent or non-virulent Agrobacterium tumefaciens or Agrobacterium rhizogenes strains which contain the binary plasmid pGA472 or PGA482 or their deriv-

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atives. Both are available from Dr. G. An, Washington State University, Pullman, WA. This binary plasmid encodes a plant expressible NPT II gene within its T-DNA region and their derivatives contain genes that will convey useful traits to transformed species. Most 5 plants resulting from seeds inoculated with virulent Agrobacterium strains, which also contained the binary plasmid, developed typical crown galls. However, NPT II activity was found in the leaves of some inoculated whole plants, indicating that the binary T-DNA region was also transferred. Transfer of the binary T-DNA region was also 10 accomplished by using avirulent strains of A. tumefaciens or rhizogenes. Results presented here show that 1.6% of the P. vulgaris and about 1% of the Glycine Max (soybean) plants were transformed, with transformation being determined by the presence of NPT II enzyme 15 activity.

Seeds of Phaseolus vulgaris cv. Olathe or Glycine max (cv.A0949) were surface sterilized with 15% Clorox for 10 minutes, followed by 4-5 rinses with distilled water and then placed on moistened paper towels in a temperature controlled Percival incubator at 28°C. and allowed to germinate for various times, 16 to 96 hours. Seed coats 20 were removed and the decoated seeds were opened in halves (that is how cotyledons were removed from the main seed body). The mesocotyl region of the germinating seeds, with their plumule still attached, were infected with an overnight liquid culture of various Agrobacterium strains by using an Eppendorf pipetter fitted with a 27 1/2 gauge needle. Seeds were infected with virulent or avirulent A. tumefaciens strains (A208, C58, C58z707 and A208/phas-zein) or A. rhizogenes strains [A4RS and A4RS(pR:B278b)pu3.3c-1]. The common A. tumefaciens and A. rhizogenes strains are available from ATCC, 12301 25 Parklawn Drive, Rockville, MD. The disarmed A. rhizogenes strain RS(pRiB278b) has been described by Vilaine and Casse-Delbart (1987) Mol. Gen. Genet., 206,17 and is available from Dr. F. Casse-Delbart, C.N.R.A., Routede Saint Cyr, F78000, Versailles, France. The disarmed A. tumefaciens, strain C582707 is available from Dr. A. G. Hepburn, University of Illinois, Urbana, IL. Inoculated seeds were 30 then placed on moistened paper towels in petri dishes and incubated at 28°C. After four days these seedlings were transplanted to soil and grown to maturity in the greenhouse. Plants infected with 35 virulent strains of A. tumefaciens were scored for efficiency of gall

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formation as a function of germination time.

NPT II Enzyme Activity

NPT II enzyme activity was detected by the *in situ* gel assay as reported by Reiss et al. (1984). Briefly, 100 mg. of a leaf tissue was mixed with 20 ml. of extraction buffer in a 1.5 ml. Eppendorf tube. Tissue samples were macerated with a Konte pestle and centrifuged for 20 minutes at 4°C. A 35 µl aliquot of the supernatant solutions was electrophoresed on a non-denaturing 10% polyacrylamide gel. The gel was overlaid with a 1% agarose gel containing 67 mM. tris-maleate (pH 7.1), 42 mM. MgCl<sub>2</sub>, 400 mM NH<sub>4</sub>Cl, 20 µg kanamycin sulfate and 200 µCi *gamma*-[<sup>32</sup>P]ATP. After incubating for 30 minutes at room temperature, the agarose gel was blotted onto Whatman P81 phosphocellulose paper overnight. The P81 paper was removed, washed several times with hot water (80°C.) and autoradiographed.

The following examples utilize many techniques well known and accessible to those skilled in the arts of molecular biology and manipulation of *Agrobacterium* strains and plasmids (virulent, avirulent, *cis*- or *trans*- configurations). Enzymes are obtained from commercial sources and are used according to the vendor's recommendations or other variations known to the art. Reagents, buffers and culture conditions are also known to those in the art. General references containing such standard techniques include the following: R. Wu, ed. (1979) *Meth. Enzymol.* Vol. 68; J. H. Miller (1972) *Experiments in Molecular Genetics*; T. Maniatis et al. (1982) *Molecular Cloning; A Laboratory Manual*; and D. M. Glover, ed. (1985) *DNA Cloning* Vol. II, all of which are incorporated by reference.

The purpose of these examples is to show that gene constructions exist, either constructed by us or others, which when transferred, integrated, and expressed in a plant will convey a useful trait to that plant.

Example 1

Germinating *P. vulgaris* and *G. max* seeds were inoculated about 24 hours after germination with virulent and avirulent *Agrobacterium* strains which contained modified pGA482G [constructed by clearing the *SalF* fragment from pWP866 which contains the gene for gentamycin-(3)-N-acetyl-benferose III, and is available from W. Piepersberg, P-8080, Munich, Federal Republic of Germany, into one of the *SalI* sites in pGA482, based binary vector constructions pPhas-zein [which contains

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the corn beta-zein gene (Pedersen et al., 1987 and is available from Dr. B. Larkins, Purdue University, West Lafayette, IN) transcriptionally linked to the P. vulgaris seed storage protein gene promotor (Slightom et al., 1983) or pu3.3c-1 [which contains the phaseolin 5 minigene construction (Chee et al., 1985) and is available from Agrigenetics Corp, Madison, WI]. Physical maps of these binary plasmids are presented in Chart 2.

Transfer and expression of the plant expressible NPT II gene contained within the T-DNA region of pGA482G (An et al., 1984) was 10 determined by removing two to three young leaves (usually obtained 10 inches or more above the wound site resulting from inoculating the germinating seeds), extracting the soluble proteins and testing for NPT II activity. From a total of 695 plants tested only 11 plants showed NPT II activity in these protein extracts. They are listed in 15 Table II and the NPT II positive results are shown in Chart 2. About 1.6% of the surviving inoculated seeds show NPT II activity, suggesting that the T-DNA region of the binary plasmid pGA482G is integrated in the genome of these P. vulgaris plants.

Other procedures, well known to those skilled in the art, such 20 as microinjection and high-velocity microprojectiles, can be used to transfer DNAs into the mesocotyl region and that transformed plants should result.

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TABLE I  
Frequency of Gall Formation on Seedlings Inoculated  
With the Agrobacterium Strain A208

	<u>Germination Periods</u>	<u>Frequency of Gall Formation</u>
5	6 hours	0
	12 hours	0
	24 hours	80
	36 hours	70
	48 hours	40
	72 hours	10
	90 hours	10

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TABLE II  
NPT II Positive Transformed Plants

<u>Plant Number</u>	<u>Binary Construction</u>	<u>Gall</u>
5	40 C58/phas-zein	+
	41 C58/phas-zein	+
	46 C58/phas-zein	+
	61 C58/phas-zein	-
	65 C58/phas-zein	-
10	151 C58/phas-zein	+
	258 A4RS(pR:B278b)pu3.3c-1	-
	269 A4RS(pR:B278b)pu3.3c-1	-
	296 A4RS(PR:B278b)pu3.3c-1	-
	470 A208/phas-zein	-
	552 C58Z707/phas-zein	-

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Example 2

Construction of a micro-Ti plasmid for the expression of a phaseolin mini-gene. The transfer and expression of this gene will increase the level of seed storage protein in the transformed plant.

5       2.1

Using the P. vulgaris seed storage protein gene, phaseolin, and its cDNA counterpart a mutant phaseolin gene lacking its five introns was constructed. This mutant phaseolin gene (phas-minigene) retains its natural 5' and 3' plant-regulatory sequences and the construction 10 of this plasmid (pPv3.3-cDNA) has been described by Chee et al. (1986) Gene 41:47 and Cramer et al. (1985) Proc. Natl. Acad. Sci. 82;334 and is available from Agrigenetics Corp. Madison, WI. Plasmid pPv3.3-cDNA was subjected to restriction enzyme digests; BamHI and HindIII and a 3.6 kb fragment was removed and cloned into BglII and 15 HindIII sites of the binary vector pGA482 (An et al. (1985) EMBO. J. 4:277). This construction places this mutant phaseolin gene within the right and left borders of the binary plasmid, now referred to as pμ3.3c-1, and along side of the plant expressible NPT II gene which is used for selection and identification of transformed plants. The 20 structure of binary plasmid pμ3.3c-1 is shown in Chart 1.

2.2 Use of pμ3.3c-1

This binary plasmid has to be transferred into various Agrobacterium strains, i.e. A208, C58, C58:707, LBA4404 and A4RS, etc. The method described here can be used to transfer the binary plasmid 25 pμ3.3c-1 into various plant species (e.g., common bean, soybean and other large seeded plants). In addition, multiple copies of the phaseolin minigene can be placed into the binary plasmid by sub-cloning the NcoI to BamHI fragment (3 kb fragment) from pPv3.3-cDNA into NcoI BamHI digested clone pPr 8.8 g (available from J. Slightom, The Upjohn Company, Kalamazoo, MI) which replaces the genomic part with the cDNA region of pPv3.3-cDNA. This cloning 30 experiment results in obtaining subclone pPv8.3-cDNA which contains an upstream BglII site (Slightom, et al. (1983) Proc. Natl. Acad. Sci., 80:1897) which allows for the isolation of a BglII-BamHI 3.3,5 kb fragment which was recloned into the BamHI digested plasmid 35 pPv3.3-cDNA. The orientation of the new phaseolin insert(s) can be checked and only those in the 5' and 3' orientation with respect to the first phaseolin gene are used for additional insertions. Because

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only the 3' BamHI site was retained (the BglII/BamHI ligated site is not digestible by either enzyme) this step could be repeated any number of times, depending on plasmid stability and ability to still transform E.coli and Agrobacteria. This procedure was repeated to 5 obtain as many as four phaseolin gene inserts, which were cloned using a HindIII and BamHI digest into the binary plasmid pGA482G. Having a series of these plasmids with different numbers of phaseolin genes (this can also be referred to as gene family transfer since a 10 family of similar genes is transferred in a single event) will increase the level of storage proteins in seeds of transformed plants.

### Example 3

The purpose of this example is to incorporate a modified seed storage protein which encodes a higher percentage of sulfur-containing amino acids; such a gene is referred to as High Sulfur Storage Protein (HSSP)-gene. This gene is constructed so that it is developmentally expressed in the seeds of dicotyledonous plants; this has been accomplished by using the phaseolin promoter. The modified gene must encode a substantial number of sulfur-containing amino acids. 20 Naturally occurring HSSP-genes can also be used. The two best naturally occurring HSSP-genes are the beta zein gene (15 kD) (Pedersen et al (1986) J. Biol. Chem. 201:6279) and the Brazil nut protein (Altenbach et al. (1987) Plant Mol. Bio. 8:239). However, any other natural or synthetic gene derivative of an HSSP-gene can be used for 25 the improvement of the nutritional value of seeds.

#### 3.1 Construction of a HSSP-gene

The construction of the zein derivative HSSP-gene uses the phaseolin gene promoter from clone pPv8.8-Bg [constructed by doing sight specific modification of pPv8.8g. The BglII to XbaI fragment for pPv8.8g was cloned into M13mp 17 (commercially available) to obtain clone as 13mp18Pv1.6. This was then used to produce single-stranded DNA which was annealed to an oligomer (30 residues) which contained a two-base pair change from the original phaseolin promoter region. The sequence of the oligomer was 5'CATCATAGTAGATCTAGTATT- 30 GAATATGAG-3' (opposite to coding strand). After annealing DNA. 35 polymerase I (Klenow fragment) was added and the remaining opposite strand of M13MP18pvl.6 was synthesized. The mutant M13 clone, containing a new Bgl site 7 bp from the translation start site

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(Slightom et al., 1983, ibid) of the phaseolin gene, was screened using the 32p-labeled oligomer and differential temperature hybridization. Cloned candidates were further analyzed by doing Bgl II digestions and agarose gel electrophoresis to identify particular clones containing the extra Bgl II site, the appearance of the Bgl II to Bgl II 800 bp fragment. The modified clone ml3 mp181.6 30.12.3 was isolated and DNA was isolated. From the isolated DNA an NcoI to XbaI fragment was removed and cloned into NcoI and the partial XbaI digested p 8.8g. The new clone containing the phaseolin promoter on a 800 bp Bgl II to Bgl II fragment was designated p Pv8.8g Bg.] to ensure proper expression and at a level expected for a seed storage protein, and the beta-zein clone pZG15RX (Pedersen et al., ibid). The phaseolin promoter was made accessible by a site specific mutation at position -7 which resulted in a BglII site, thus the phaseolin promoter could be removed after a BglII digest as an 800 bp fragment. This fragment was subcloned into the BamHI site of pUC18 (available from commercial sources), yielding a plasmid designate pUC-Pvpro. The beta-zein structural gene, including signal peptide, coding region, and Poly (A) addition signal was removed from plasmid pZG15EX (available from B. Larkins, Purdue University, West Lafayette, IN) after a TagI digestion and this fragment was cloned into the AccI site of pUC-Pvpro, yielding clone pUC-Phas-zein. This Phas-zein gene was removed by digestion with HindIII and EcoRI and this fragment was cloned into the binary vector pGA482G, which had previously been digested with HindIII and EcoRI. This new binary plasmid is referred to as pGA482G-Phas-zein (see Chart 2) and it was transferred into Agrobacterium strains: A208, C58, LBA4404, C58Z707, and A4RS which in turn can be used to produce transformed plants in accordance with the method of this invention.

A phase zein construction similar to that described above has been transferred into dicotyledonous plants and its developmental expression in the seeds of the transformed plant has been observed; see Hoffman et al. (1987) EMBO J. 6:3213. Additional modification has been made to a Phas-zein gene construction. These modifications include the ligation of a BglII linker onto its 5'-end and a BamHI linker onto its 3'-end which allows the construction of multiple copies of the phase zein gene as described above for the phaseolin minigene. This allows for the transfer of a HSSP-gene multigene

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family into a plant species by a single transformation event and the expression of higher levels of the HSSP-gene product. This leads to the development of dicotyledonous plant varieties which are nutritionally improved, such as common bean, soybean and other large seeded plants.

5           **Example 4 Transfer of Viral Resistance**

The purpose of this example is to generate a construction for the expression of a plant virus coat protein gene which, when expressed in a dicotyledonous plant, results in reduced symptoms or 10 resistance to later infections by that virus (see report by Powell-Abel et al. (1986) Science 232:738). Viral coat proteins are isolated from any number of plant virus classes (tobamo, cucumo, poty, tobra, AMV, etc.) and they are expressed constitutively in plants after the attachment of the CaMV 35S promoter. In addition, a 15 plant poly (A) signal is added to the 3' region to ensure proper expression.

A clone containing any specific viral coat protein gene can be obtained for both plant DNA and RNA viruses. Such is the case for cucumber mosaic virus strain C (CMV-C); its RNA genome was copied 20 into double-stranded cDNA and the coat protein gene was isolated and characterized as follows. A residues were added to the 3' end of CMV-C total RNA, using E. coli polyadenylose. This poly (A) region was used to anneal an aligo dT primer which was used to prime the synthesis of single-stranded (SS) cDNA using reverse transcriptos and 25 appropriate buffer of CMV-C SS-cDNA, double-stranded cDNA was synthesized by adding RNase H to remove the RNA from the duplex and the second strand was made by adding E. coli DNP polymerase I (Klenow fragment) and the appropriate buffer. After synthesis of CMA-C ds-DNA, it was E. coli methylated using Eco RI methylase and Eco 30 methylent buffer, thus protecting all internal Eco RI sites in the CMV-C ds-cDNA molecules. After Eco methylation the CMV-C ds-cDNA molecules were treated again with E. coli polymorse I (Klenow fragment) to ensure that all ends (5' and 3') were flush, then these molecules were ligated to Eco RI linkers using T4-Ligase. After 35 ligation the CMV-C ds-cDNA molecules were separated from contaminating linker by size fractionation on a GYOG column (1cm X 30cm). The fraction containing the majority of the CMV-C ds-cDNA molecules was EtOH precipitated, followed by resuspension in 10 µg of H<sub>2</sub>O.

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About 100  $\mu$ g of these Eco RI linked CMV-C ds-cDNA molecules were removed and mixed with 1 $\mu$ g of  $\lambda$  gT11 arms (commercially available) and ligated together using T4 ligase. The recombinant GT 11-CMV-C were plated using E. coli Up50supF as host and these plates ( $10^{-4}$  clones) were screened for clones containing CMV-C coat protein gene coding region using p-labeled CMV-whiteleaf SS-cDNA as probe. From this screening, a clone,  $\lambda$  GT11-CMV9.9 was isolated. It contained an EcoRI insert of 1400 base pair, enough to encode the complete CMV coat protein. This CMV coat protein gene can be expressed in plant tissues once a plant-active promoter and poly (A) signal are attached to its 5' and 3' regions, respectively. The scheme to accomplish this is shown in Chart 3.

Attachment of the constitutive cauliflower mosaic virus (CaMV) 35S promoter was done by first doing a partial AccI and complete EcoRI digests of clone pCMV9.9 which was obtained by cloning the Eco RI insert from Lambda GT11-CMV9.9 into EcoRI cut puc 19 (commercially available). The 1100 bp CMV-C coat protein gene fragment was removed, both ends were blunted, and this fragment was cloned into the SmaI site of pDH51 (Pietrzak et al. (1986). Nuc. Acids Res. 14:5857) which is available from A.T. Mohn, Friedrick Mieschen Institut, Basel, Switzerland to obtain clone pDH51/cP19.. This positioned the CMV-C coat protein gene downstream of the CaMV 35S promoter and upstream from the CaMV poly (A) signal sequence. To ensure a high level of expression other poly (A) signal sequences (which may function better than the CaMV 35S poly (A) signal) can be attached, such as the poly (A) signal from the seed storage protein gene phaseolin (Slightom et al. (1983) Proc. Natl. Acad. Sci. 80:1897). To facilitate engineering, this plant expressible CMV-C coat protein gene was removed from clone pDH51/CP19 by an EcoRI digest and the 1800 bp fragment was cloned into pUC1813 (which contains more restriction enzyme sites and is available from Dr. R. Kay, Washington State University, Pullman, Washington. The resulting clone, pUC1813/CP19, was then partially digested with HindIII and the 1800 bp fragment was cloned into the binary vector pGA482 to obtain the new clone, pGA482/CP19H (see Chart 3). This binary plasmid, or its derivatives, can be transferred into Agrobacterium strains: A208, C58, LBA4404, C58Z707, A4RS, A4RS(pRIB28b) and others. Using the transformation method of this invention, this plant expressible CMV-C

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coat protein gene (or any other plant virus coat protein gene) can be transferred into a dicotyledonous plant species such as, cucumber, squash, melon, zucchini, pepper, etc. The development of these new cultivars are useful because of their resistance to infections by specific virus or viruses (if more than one virus coat protein gene construction is transferred to a single plant).

Example 5 Transfer of Herbicide Resistance

The purpose of this example is to illustrate how to generate plant expressible genes which allow a plant to be resistant to specific classes of herbicides. Such plants are useful for many reasons; (i) herbicides normally lethal can be used, and (ii) different crops can be used in close rotations on soil which may contain residual amounts of a previously used herbicide that is normally lethal to the second crop. Two genes of interest are mutant derivatives (derived from plant or bacterial sources) of the aceto-lactate synthase (ALS) gene which are not sensitive to chlorsulfuron and sulfometuron methyl herbicides (Falco et al., (1985) Biotech. Plant Sci. Academic Press, Inc. page 313) and mutants of the gene encoding enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Stalker et al., (1985) J. Biol. Chem., 260:4724) which are not sensitive to the herbicide glyphosate.

A gene which encodes an important enzyme which is either resistant to or detoxifies a specific herbicide is cloned downstream from a plant active promoter, such as: CaMV 35S, ribulose-1,5-bisphosphate carboxylase small subunit gene, or other strong plant gene promoter and upstream from a plant gene poly (A) signal sequence, see Chart 4.

This gene is then be cloned into an Agrobacterium-derived vector (either binary or cis) and using the above-described plant transformation method, such a gene is be transferred into many dicotyledonous plant species, such as: soybean, common bean, peppers, melons, etc.

Example 6 Transfer of Insect-Resistant Gene

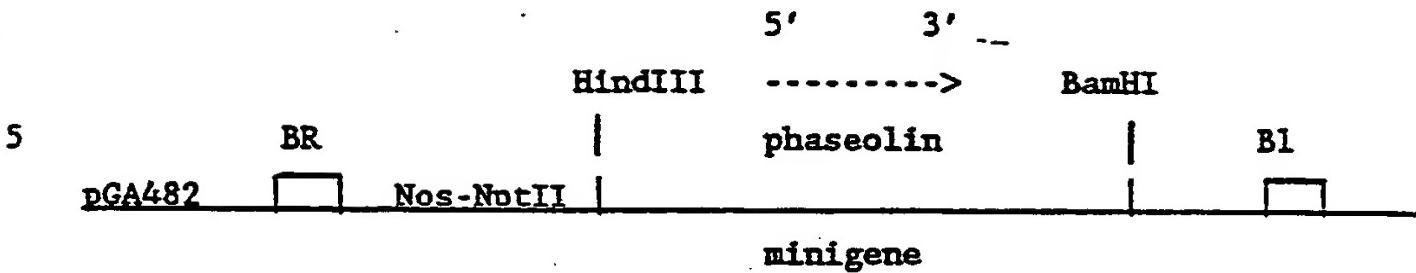
In nature, numerous polypeptides exist which are toxic to insect pests. The best known protein toxins are those associated with different strains of Bacillus thuringiensis; for example, B. israelensis active against Diptera (mosquitoes and blackflies), B. thuringinensis active against Lepidoptera, and B. san diego active against Coleoptera. The toxin protein found in each of these bacteria is highly specific to insect pests; they are not toxic to other

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organisms. Thus the transfer and expression of genes encoding such toxic proteins in plants are beneficial in reducing insect damage without using chemical insecticides thereby avoiding risk to other organisms. The genes encoding many of these toxic proteins have been 5 isolated and sequenced (Schnepp et al. (1985) J. Biol. Chem., 260:6264; Waalwijk et al., (1985) Nucl. Acids Res., 13:8207; Sekar et al (1987) Proc. Natl. Acad. Sci., 84:7036). The transfer of the B.thuringiensis toxic gene into tobacco and its usefulness in protecting the plant from insect damage has been reported (Vaeck et 10 al. (1987) Nature 328:33). Thus, the combination of using the plant transformation system described here and plant expressible Bacillus toxin gene (see Chart 5) allows for the transfer of a useful trait to any dicotyledonous species for which tissue-culture based transformation systems are inefficient or have not been developed, such as: 15 common bean, soybean, melon, cucumber, squash, zucchini, pepper, etc.

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**Chart 1**



10

**Chart 2**

15

3' 5'

BR

Plant

phase

BT

R

pGA4826

### Nos-NptII

zein

Gent

20

Poly(A)

四三二

25

**Chart 3**

30

5'                    3'

3

plant

**poly(A) signal**

BR

CMV - C

BR

35

promoter from other plant viruses

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Chart 4

5' \_\_\_\_\_ 3'

5

BR	Plant	Mutant	Plant	BL
pGA482	<input type="checkbox"/> Nps-NptII   Promoter	ALS or EPSPS gene	Poly(A) <input type="checkbox"/>	
		or other herbicide		
		resistant or detoxgene		

10

Chart 5

5'            3'

----->

15

BR	Plant	Bacillus or	Plant	BR
pGA482	<input type="checkbox"/> Nos-NptII   Promoter	other toxgene	Poly(A) <input type="checkbox"/>	
		for insect		
		pest		

20

25

30

35

-20-

CLAIMS

1. A process for producing a transgenic plant which comprises:
  - (a) germinating a seed of a plant;
  - 5 (b) inoculating the meristematic or mesocotyl cells produced during germination, prior to their differentiation, with a virulent or non-virulent Agrobacterium strain containing a transferable gene in an Agrobacterium derived vector; and
  - (c) allowing the cells to differentiate into mature plants,
- 10 with the proviso that the plant cannot be from the family Arabidopsis thaliana.
2. A process according to claim 1 wherein the vector is a plasmid adapted for either transfer in trans- or cis- configuration.
- 15 3. A process according to claim 2 wherein the vector is a binary plasmid adapted for transfer in the trans configuration.
4. A process according to claim 3 wherein the plant is dicotyledonous.
- 20 5. A process according to claim 4 wherein one of the cotyledons is removed prior to inoculation.
- 25 6. A transgenic plant prepared by the process of claim 1.
7. A transgenic dicotyledonous plant according to claim 6.
8. A plant according to claim 7 wherein the plant is a member of the family leguminosae.
- 30 9. A plant according to claim 8 wherein the plant is a soybean.
10. A plant according to claim 9 wherein the plant is common bean.
- 35 11. A plant according to claim 10 wherein the plant gene is the gene for phaseolin.

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US 88/04464

## I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) \*

According to International Patent Classification (IPC) or to both National Classification and IPC

**IPC<sup>4</sup>**: C 12 N 15/00; A 01 H 1/00

## II. FIELDS SEARCHED

Minimum Documentation Searched \*

Classification System	Classification Symbols
IPC <sup>4</sup>	C 12 N; A 01 H

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched \*

## III. DOCUMENTS CONSIDERED TO BE RELEVANT \*

Category *	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	FR, A, 2560744 (PHYTOGEN) 13 September 1985, see the whole document	1
Y	Mol. Gen Genet, vol. 208, no. 1/2, June 1987, Spring-Verlag K.A. Feldmann et al.: "Agrobacterium- mediated transformation of germinating seeds of <i>Arabidopsis thaliana</i> : A non- tissue culture approach", pages 1-9 see the whole document	1-10
X	Plant Mol. Biol., vol. 8, no. 3, 1987, M. Nijhoff Publishers, Dordrecht (NL) K.Sukhapinda et al.: "Ri-plasmid as a helper for introducing vector DNA into alfalfa plants", pages 209-216 see abstract	6-8
O,X	Biological Abstracts/RRM, no. 89:116856 T.C. Hall et al.: "Transformation of plant cells", see abstract 36:62272 & Ciba Foundation Symposium, no. 137, Applications of plant cell and tissue	6-8,10

\* Special categories of cited documents: <sup>10</sup>

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the International filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

11th April 1989

Date of Mailing of this International Search Report

4. 05. 89

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

M. VAN MOL

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
O,X	culture; symposium, Kyoto, Japan, 20-22 October 1987, IX+269P. John Wiley and Sons, Inc.: Somerset, NJ US, Chichester, GB Illus. 0 (0), 1988, 123-138 Biological Abstracts/RRM W. Lin et al.: "Soybean tissue culture and genetic transformation" see abstract 33117694 & Int Bot Congr Abstr 1987. vol. 17, no. 0, p167, --	6-9
O,X	J. Cell Biochem. Suppl. 11B, S.L. Goldman et al.: "Transformation of Zea mays by Agrobacterium tumefaciens: Evidence for stable genetic alterations", page 26, see abstract F 202 --	1,2,6
P,X	Chemical Abstracts, vol. 109, 1988, (Columbus, Ohio, US) see page 193, abstract 105884p & JP, A, 6387921 (UNIVERSITY OF TOLEDO) 19 April 1988 --	1-3,6
P,X	EP, A, 0267159 (CIBA-GEIGY & LUBRIZOL GENETICS) 11 May 1988, see claims --	1-10
P,X	EP, A, 0256751 (LUBRIZOL GENETICS) 24 February 1988, see the whole document --	6-9
A	EP, A, 0241963 (C.E.N.) 21 October 1987, see the whole document --	1-10
A	Plant. Molecular Biology, vol. 7, 1986 M. Nijhoff Publishers, Dordrecht (NL) A.C.F. Graves et al.: "The transformation of Zea mays seedlings with Agrobacterium tumefaciens" pages 43-50 see the whole document --	1-10
A	Nature, vol. 325, no. 7000, 7-14 January 1987, (Neptune, NJ, US) N. Grimsley et al.: "Agrobacterium-mediated delivery of infectious maize streak virus into maize plants", pages 177-179, see the whole document --	1-10

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	EP, A, 0064720 (RESEARCH AND DEVELOPMENT INSTITUTE INC. MONTANA) 17 November 1982, see exemple 13; table 13 --	10
E	EP, A, 0301749 (AGRACETUS) 1 February 1989, see example 8	6-9

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

US 8804464  
SA 25950

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 16/05/89. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
FR-A- 2560744	13-09-85	None		
EP-A- 0267159	11-05-88	AU-A-	8089387	12-05-88
		JP-A-	63141590	14-06-88
EP-A- 0256751	24-02-88	AU-A-	7654187	11-02-88
		JP-A-	63177795	21-07-88
EP-A- 0241963	21-10-87	JP-A-	62253327	05-11-87
		LU-A-	86372	11-11-87
EP-A- 0064720	17-11-82	AU-A-	8322982	18-11-82
		JP-A-	58023782	12-02-83
		US-A-	4425150	10-01-84
		AU-B-	537644	05-07-84
		CA-A-	1183361	05-03-85
		US-A-	4517008	14-05-85
EP-A- 0301749	01-02-89	AU-A-	2019688	02-02-89